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Influenza H7N9 and H9N2 Viruses: Coexistence in Poultry Linked to Human H7N9 Infection and Genome Characteristics

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ABSTRACT

Avian influenza virus A of the novel H7N9 reassortant subtype was recently found to cause severe human respiratory infections in China. Live poultry markets were suspected locations of the human H7N9 infection sources, based on the cases' exposure histories and sequence similarities between viral isolates. To explore the role of live poultry markets in the origin of the novel H7N9 virus, we systematically examined poultry and environmental specimens from local markets and farms in Hangzhou, using real-time reverse transcription-PCR (RT-PCR) as well as high-throughput next-generation sequencing (NGS). RT-PCR identified specimens positive for the H7 and N9 genomic segments in all of the 12 poultry markets epidemiologically linked to 10 human H7N9 cases. Chickens, ducks, and environmental specimens from the markets contained heavily mixed subtypes, including H7, N9, H9, and N2 and sometimes H5 and N1. The idea of the coexistence of H7N9 and H9N2 subtypes in chickens was further supported by metagenomic sequencing. In contrast, human H7N9 infection cases ($n = 31$) were all negative for H9N2 virus according to real-time RT-PCR. The six internal segments were indistinguishable for the H7N9 and H9N2 viruses. The H9, N2, and internal-segment sequences were very close to the sequence of the H9N2 virus circulating in chickens in China recently. Our results provide direct evidence that H9N2 strains coexisted with the novel human-pathogenic H7N9 influenza virus in epidemiologically linked live poultry markets. Avian influenza A virus of the H9N2 subtype likely made a recent contribution to the evolution of the H7N9 virus and continues to do so.

IMPORTANCE

Our results suggest that avian influenza A virus of the H9N2 subtype likely made a recent contribution to the evolution of the H7N9 virus, a novel reassortant avian influenza virus A subtype, and continues to do so. The finding helps shed light on how the H7N9 virus emerged, spread, and transmitted to humans. It is of considerable interest for assessing the risk of the possible emergence of novel reassortant viruses with enhanced transmissibility to humans.

Avian influenza virus A of novel reassortant subtype H7N9 was found to cause severe human respiratory infection in Shanghai and Anhui Province, eastern China, in March 2013 (1). As of 20 May, a total of 130 laboratory-confirmed H7N9 infection cases had been reported in 10 municipalities or provinces across China, 36 of which were fatal. Bioinformatic analyses for the H7N9 virus revealed that its eight genomic segments had at least four possible origins: the hemagglutinin (HA) gene from avian influenza virus of duck origin, the neuraminidase (NA) gene from migratory bird, and the six internal genes from two different groups of H9N2 avian influenza viruses from chickens (2). It is believed that the sources of the H7N9 human infections were linked to the local live poultry markets based on the cases' exposure histories and the high similarities between viral RNA sequences from humans and poultry markets (3, 4, 5), although some of the cases had no apparent history of poultry exposure. The live poultry markets in China bring together many types of poultry under conditions of high density, providing an ideal environment for interspecies transmission and evolution of avian influenza viruses (6).

For Hangzhou, Zhejiang Province, which is close to Shanghai Municipality, the H7N9 virus has been identified in specimens from human cases as well as from local poultry markets (3). To explore the role of live poultry markets in the origin of the novel H7N9 virus, here we investigated the existence of the avian influenza viruses among the specimens from live poultry and environments at local markets and farms using real-time reverse transcription-PCR (RT-PCR) and metagenomics based on next-generation sequencing (NGS). We found heavy contaminations

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TABLE 1 The existence of influenza A virus subtypes H7, H9, H9, N2, H5, and N1 in specimens collected from live poultry markets and chicken farms in Hangzhou, Zhejiang province, China, determined by real-time RT-PCR

Source	Linkage to H7N9 infection case	Specimen(s)[a]	Collecting date	No. for testing	No. with different H and N patterns[b]					
					H7+N9+, H9+N2+, H5+N1+	H7+N9+, H9+N2+, H5−N1−	H7+N9+, H9−N2−, H5−N1−	H7−N9−, H9+N2+, H5−N1−	H7+N9+, H9−N2−, H5+N1+	H7−N9−, H9−N2−, H5−N1−
Live poultry markets	Yes	Pharyngeal and rectal swabs from chicken	From 2 markets on 4 and 10 April	15	6	4	0	0	0	5
		Pharyngeal and rectal swabs from duck	From 1 markets on 4 and 10 April	8	0	2	2	0	0	4
		Pharyngeal swab from quail	From 1 market on 4 April	1	0	0	0	1	0	0
		Environmental specimens	From 12 markets during 4 to 20 April	86	19	27	6	4	2	28
Total				110	25	33	8	5	2	37
Live poultry markets	No	Environment specimens	From 5 markets on 17 and 18 April	30	6	4	3	0	0	17
Chicken farms	No	Pharyngeal swabs from chicken	From 2 farms on 12 April	15	0	0	1	0	0	14
		Environment specimens	From 8 farms on 12, 16, 17, 18, and 19 April	96	0	0	2	0	0	94
Total				111	0	0	3	0	0	108

[a] Environmental specimens = poultry feces, sewage, water for shedding feather, smears from surfaces of cages, feather and vessel, etc.
[b] +, positive; −, negative.

of both H7N9 and H9N2 viruses, and sometimes even H5N1 virus, simultaneously in the poultry and environments at local live poultry markets epidemiologically linked to the human H7N9 infection cases in Hangzhou. The H9, N2, and internal-segment sequences were very close to those of the H9N2 virus circulating in chickens in China recently. The results provided direct evidence that avian influenza A virus of the H9N2 subtype likely made a recent contribution to the evolution of the H7N9 virus and that the contribution is ongoing.

MATERIALS AND METHODS

Specimen collection. The first H7N9 virus infection case in Zhejiang, China, was identified in Hangzhou, the capital of Zhejiang province, on 1 April 2013 (3, 7). Since then, whenever an H7N9 infection case was laboratory confirmed in Hangzhou, an epidemiological investigation was initiated for the infected subject. From the live poultry markets epidemiologically linked to the human cases, specimens of poultry feces, poultry pharyngeal swabs, poultry cage swabs, and water for shedding feather (see Table 1; see also Table S1 in the supplemental material) were collected for detecting avian influenza A virus subtypes H7, N9, H9, N2, H5, and N1 by real-time RT-PCR. All of these markets were scattered among urban areas in Hangzhou, which are illustrated in Fig. 1, with one exception located at a village 77 kilometers west of Hangzhou. Environmental specimens from local poultry farms and live poultry markets that were not linked to the human H7N9 infection cases were also collected as controls. The patient's respiratory specimens (including throat swab, sputum, or nasopharyngeal aspirate specimens) were collected and transferred to our laboratory to test the nucleic acids of H7N9 virus and H9N2 virus.

RT-PCR screening of H7N9 and H9N2 in human and poultry specimens. Viral RNA was extracted from the specimens using an RNeasy Minikit (Qiagen, Germany). Identification of the avian influenza A virus was achieved by real-time RT-PCR targeting segments M (for all influenza A viruses), H7, N9, H9, H5, and H1, according to the protocol provided by the WHO Collaborating Center for Reference and Research on Influenza at the Chinese National Influenza Center, Beijing, China. The primers and TaqMan probe for N2 real-time RT-PCR detection were provided by Shanghai Chaoshi Biotechnology Company, China.

Next-generation sequencing. The quality and integrity of the RNA samples were checked using an Agilent Technologies 2100 Bioanalyzer. RT-PCR was conducted with SuperScript II reverse transcriptase (Invitrogen). First-strand cDNA was synthesized with the influenza A virus-specific primer MBTuni-12 (Invitrogen) (8) in a 20-μl reaction volume according to the manufacturer's protocol. The RT reaction was performed at 42°C for 50 min. To obtain full-length amplification of all influenza A viruses, viral RNA segments were separately amplified with an universal primer set for the eight viral RNAs of influenza A viruses, using KOD high-fidelity DNA polymerase according to the standard protocol (Toyobo). The primer sequences have been published previously (9). One microliter of the RT reaction mixture was used as the template for the PCR. The PCR conditions comprised an initial denaturation at 94°C for 2 min; 35 cycles of 98°C for 10 s and 68°C for 2.5 min; and a final extension at 68°C for 5 min. The full-length PCR amplicons of eight viral RNA segments were pooled to construct a DNA library for high-throughput sequencing for each sample. The pooled PCR products were fragmented by sonication in a Covaris LE220 Focused-ultrasonicator (Covaris). The DNA fragments were then processed by end repairing, A-tailing, adapter ligation, DNA size selection, PCR amplification, and product purification according to the instructions of the manufacturer (Illumina). The DNA libraries with an insert size of 300 bp were sequenced by 150-bp paired-end sequencing on an Illumina MiSeq Personal Sequencer.

Sequencing data assembly and bioinformatic analysis. NGS reads were cleaned by filtering out low-quality reads (eight reads with quali-

FIG 1 The locations of the live poultry markets epidemiologically linked to the H7N9 patients in Hangzhou, China. The abbreviations on the map at the upper right represent the names of the live markets epidemiologically linked to the H7N9 patients in Hangzhou. The market of JSC is not shown on the right upper portion of the map due to its location at a village 77 kilometers west of Hangzhou.

ties < 66), duplication, poly-Ns (with eight Ns), adaptor-contaminated reads (with >15 bp matched to the adapter sequence), and reads mapped to the host (using SOAP [10], five mismatches). The remaining high-quality reads were *de novo* assembled using SOAPdenovo (version 1.06) and edena (v3.121122) (11, 12).

The clean reads were mapped to the INFLUENZA database (13) to retrieve the best-matching reference sequences. We used BWA(0.7.3a) (14) and samtools(0.1.19) (15) to perform assembly based on the reference sequences. Wrong indels and mismatches in these reference-based assemblies were eliminated by aligning the *de novo*-assembled contigs (>200 bp) to the sequences. The improved sequence was used as a reference to reassemble the high-quality reads to generate the final reference-based assembly sequences. In addition, we performed *de novo* assembly using Vicuna (1.1) (16) software and then combined the results with the reference-based assembly to obtain the best assembly. The complete genome sequences of H9N2 and H7N9 viruses from the live poultry markets in Hangzhou were deposited in the Global Initiative on Sharing All Influenza Data (GISAID) under the accession numbers listed in Table 2.

Phylogenetic analyses. The nucleotide sequence-based phylogenetic trees were constructed via neighbor-joining method with bootstrap analysis (n = 1,000) using the MEGA5.0 program (17).

Nucleotide sequence accession numbers. All eight gene segments of H7N9 viruses and H9N2 viruses were phylogenetically analyzed separately, together with homologous sequences available in GenBank and the GISAID under the accession numbers given in Table 2 and the following additional accession numbers: JX679164.1, JF789602.1, JN244234.1, AB481213.1, HQ244409.1, GU060482.1, CY060360.1, CY122246.1, HM998922.1, HM998924.1, AF508574.1, AF508595.1, AY043019.1, AY043024.1, AF156376.1, AF156394.1, AF156377.1, AF156395.1, AF156373.1, AF156391.1, AY253750.1, AY253751.1, AY253752.1, AY253753.1, AY253755.1, AY253756.1, AY743216.1, AY253754.1, KC417068.1, KC417065.1, KC417062.1, KC417056.1, KC417050.1, KC417059.1, KC417047.1, KC417053.1, JF795091.1, JF795092.1, JF795093.1, JF795095.1, JF795097.1, JF795098.1, JF795094.1, JF795096.1, JN653558.1, JN653574.1, JN653590.1, JN653622.1, JN653654.1, JN653670.1, JN653606.1, JN653638.1, AF156435.1, AF156421.1, AF156449.1, AF156407.1, AF156463.1, AF156477.2, AF156378.1, AF156396.1, KC464595.1, KC464596.1, KC464597.1, KC464599.1, KC464601.1, KC464602.1, KC464598.1, KC464600.1, AF156438.1, AF156423.1, AF156452.1, AF156409.1, AF156466.1, AF156480.1, AF156380.1, AF156398.1, JQ356884.1, JQ356887.1, JQ356881.1, JQ356878.1, JQ356890.1, JQ356893.1, JQ356872.1, and JQ356875.1 (see also Table S2 in the supplemental material).

TABLE 2 The GISAID accession numbers of H7N9, H9N2, and H5N1 viruses from the live poultry markets[a]

Virus	GISAID accession no. for segment:												
	PB2	PB1	PA	NP	M	NS	H7	N9	H9	N2	H5	N1
A/environment/Hangzhou/34-1/2013(H7N9)	EPI442715	EPI443570	EPI443571	EPI443572	EPI443573	EPI443574	EPI442716	EPI442717	EPI473793	EPI473795		
A/environment/Hangzhou/34-2/2013(H9N2)	EPI473790	EPI473791	EPI473792	EPI473794	EPI473796	EPI473797						
A/chicken/Hangzhou/48-1/2013(H7N9)	EPI443656	EPI443657	EPI443658	EPI443660	EPI443662	EPI443663	EPI443659	EPI443661	EPI454489	EPI454490		
A/chicken/Hangzhou/48-2/2013(H9N2)	EPI468349	EPI468350	EPI468351	EPI468352	EPI468353	EPI468354						
A/chicken/Hangzhou/50-1/2013(H7N9)	EPI443664	EPI443665	EPI443666	EPI443668	EPI443670	EPI443671	EPI443667	EPI443669	EPI454491	EPI454492		
A/chicken/Hangzhou/50-2/2013(H9N2)	EPI468355	EPI468356	EPI468357	EPI468358	EPI468359	EPI468360						
A/environment/Hangzhou/109-1/2013(H7N9)	EPI443672	EPI443673	EPI443674	EPI443676	EPI443678	EPI443679	EPI443675	EPI443677			EPI454493	EPI454494
A/environment/Hangzhou/109-2/2013(H5N1)	EPI468343	EPI468344	EPI468345	EPI468346	EPI468347	EPI468348						
A/quail/Hangzhou/35/2013(H9N2)	EPI450828	EPI450829	EPI450830	EPI450832	EPI450834	EPI450835			EPI450831	EPI450833		

[a] The sequences of six internal segments between the H7N9 virus and the H9N2 virus coexisting in the same specimen [e.g., A/environment/Hangzhou/34-1/2013(H7N9) and A/environment/Hangzhou/34-2/2013(H9N2)] were indistinguishable.

RESULTS

Coexistence of H7/H9, N9/N2, and H5/N1 in poultry markets determined by real-time RT-PCR. Poultry and environment specimens were collected from 12 poultry markets epidemiologically linked to 10 human H7N9 infection cases that occurred between 4 and 20 April 2013 in Hangzhou. Specimens positive for the H7 and N9 segments were found in all 12 of the poultry markets (Table 1; see also Table S1 in the supplemental material). Coexistence of H7N9 and H9N2, even with H5N1, was detected in chickens and ducks. Among 15 pharyngeal and rectal swabs from chickens in two live poultry markets, 6 were found positive for H7, N9, H9, N2, H5, and H1 and 4 positive for H7, N9, H9, and N2. In eight pharyngeal and rectal swabs from ducks in one live poultry market, two were positive for H7, N9, H9, and N2 and two positive for H7 and N9. In one pharyngeal swab from a quail, only H9N2 was detected. The environments of these poultry markets linked to H7N9 cases were heavily contaminated by influenza A H7N9 and H9N2 viruses, with or without H5N1 virus. A total of 46 of 86 specimens (53.5%) from the environment in all 12 markets were positive for H7, N9, H9, and N2. Thus, the novel H7N9 virus likely coexisted generally with at least an H9N2 virus in live poultry markets.

Specimens from five poultry markets with no apparent epidemiological linkages to the laboratory-confirmed H7N9 human cases were also tested for these avian influenza viruses. Thirteen H7N9-positive specimens, including six positive for H7N9, H9N2, and H5N1, four positive for H7N9 and H9N2, and three for H7N9 only, were found in 30 specimens from three of these five markets.

Neither H7N9 nor H9N2 was detected in 109 specimens from poultry and/or environment in seven local farms. However, three H7N9-positive and H9N2-negative (one chicken throat swab, one cage smear, and one drinking water for chicken) specimens were found among five specimens from one local farm.

In contrast to the coexistence of H7N9 and H9N2 subtypes frequently found in poultry, the H7N9-positive specimens from 31 human H7N9 infection cases were all negative for H9N2 virus according to real-time RT-PCR.

Coexistence of multiple subtypes by metagenomic sequencing. In order to quantitatively evaluate the coexistence of H7N9 and H9N2 subtypes in poultry, we applied high-throughput next-generation sequencing to the study of influenza A virus. We generated a total of 4,411,007 paired-end clean reads for two chicken pharyngeal swabs, one quail pharyngeal swab, and six environmental samples after removing adaptor-contaminated or low-quality reads. The percentage of influenza virus-matched reads ranged from 0.55% to 80.97%, with better matches and more reads for poultry swabs than environmental samples (Table 3). The non-influenza virus reads, likely originated from nonspecific amplifications, could be mainly classified to host (*Gallus* family) or bacteria, as well as unknown sequences (data not shown). We were able to assemble complete genomes for five of the samples, including 100% open reading frames of the eight genomic segments. Only partial genomes were obtained for the other three samples due to low coverage of the viral sequences.

After mapping to the INFLUENZA database, different subtypes of influenza virus clearly coexisted in six of the samples (Fig. 2). Four of the samples contained significant amounts of both H7 and H9 and of both N2 and N9. The chicken pharyngeal

TABLE 3 General statistics for environment samples analyzed by NGS

Sample no.	Source	Live poultry market[a]	No. of clean reads	No. of reads mapped to influenza virus	No. of reads mapped to influenza virus (%)	Complete genome	Virus name
hd6	Chicken pharyngeal swab	YH	451,309	333,387	73.87	Yes	A/chicken/Hangzhou/48-1/2013(H7N9) A/chicken/Hangzhou/48-2/2013(H9N2)
hd7	Chicken pharyngeal swab	YH	457,227	266,592	58.31	Yes	A/chicken/Hangzhou/50-1/2013(H7N9) A/chicken/Hangzhou/50-2/2013(H9N2)
hd11	Quail pharyngeal swab	BS	501,666	406,195	80.97	Yes	A/quail/Hangzhou/35/2013(H9N2)
hd4	Chicken feces	BS	517,761	101,554	19.60	Yes	A/environment/Hangzhou/34-1/2013(H7N9) A/environment/Hangzhou/34-2/2013(H9N2)
hd5	Water for shedding feather	BS	637,104	13,547	2.13	No	
hd8	Poultry feces	ZLK	673,346	3,727	0.55	No	
hd9	Chicken feces	DX	554,675	193,079	34.81	Yes	A/environment/Hangzhou/109-1/2013(H7N9) A/environment/Hangzhou/109-2/2013(H5N1)
hd10	Duck feces	DX	617,919	7,309	1.18	No	
Total			4,411,007	1,325,390			

[a] The abbreviations in this column represent the names of the live poultry markets where the samples were collected.

swabs (hd6 and hd7) with an over 50% match to influenza virus displayed similar percentages of H7 and N9 and of H9 and N2, consistent with the chickens hosting influenza virus subtypes H7N9 and H9N2. One chicken feces sample (hd9) was detected as a mixture of H5 and H7 and of N1 and N9. One duck feces sample (hd10) showed more complicated subtypes with H5, H7, and H9 and N1, N2, and N9. According to the relative abundances of reads classified to different HA and NA subtypes, we concluded that H7N9, H9N2, or H5N1 was probably the major subtype of influenza virus in the specific samples. Thus, all these vRNA formed a cloud-like community.

Important amino acid characteristics in the proteins of the H7N9 and H9N2 viruses. Both H7 and H9 of the viruses from live poultry markets had the substitution of Q226L (H3 numbering) at the receptor binding site of HA (Table 4), which has been reported to contribute to the high-affinity binding of H5 and H7 viruses to the human receptor (18, 19). The sequences of cleavage sites in H7 and H9 were featured with avirulent types. A deletion in the NA stalk (positions 69 to 73), which was associated with the adaption to gallinaceous hosts (20), was found in N9 segments. In the N2 stalk, a deletion at positions 63 to 65 was identified also which was able to improve NA enzyme activity and release of virus from erythrocytes (21). R294K is believed to confer resistance to oseltamivir, and position 294 of NA remained R in both N7 and N9.

The six internal segments were indistinguishable for the H7N9 and H9N2 viruses found in chickens at live poultry markets in Hangzhou (Table 4). The substitutions of N30D and T215A in M1 and P42S in NS, implicated with increased virulence in mice, were observed in the viruses from both poultry and patients with H7N9 infection. However, the E627 in PB2 in the viruses from poultry markets contrasted with the E627K substitution found in viruses from most human patients.

Phylogenetic analyses of H7N9 and H9N2 in poultry. The H7 and N9 sequences of the H7N9 viruses from live poultry markets were very close to the H7 and N9 sequences from the clinical and environmental isolates identified recently (Fig. 3). The H9 and N2

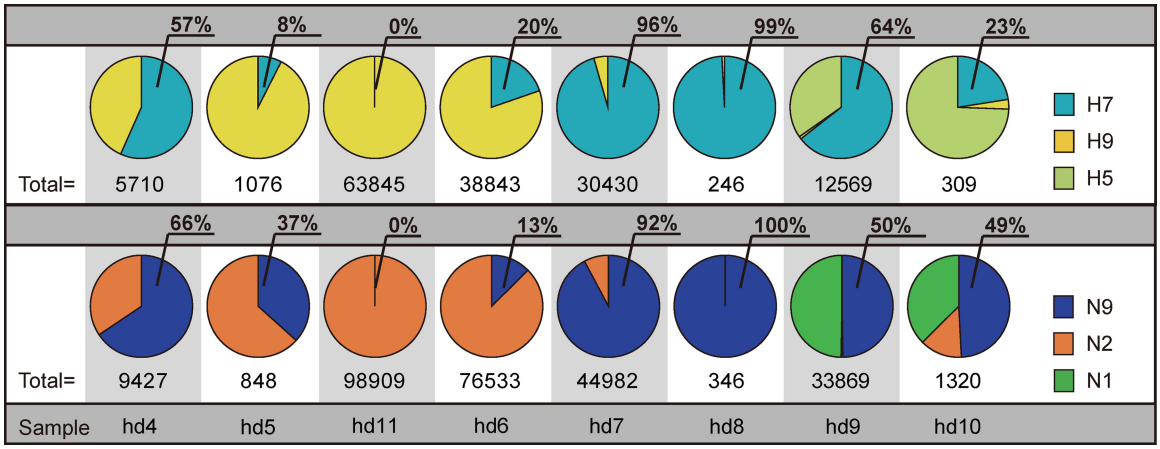

FIG 2 Subtypes of influenza A virus found in the poultry specimens. The numbers under circles represent the total reads of NGS mapping to H7, H9 and H5, or N9, N2, and N1.

TABLE 4 Important amino acid characteristics in the HA, NA, PB2, M1, M2, and NS1 proteins associated with interspecies transmission and drug resistance of H7N9 and H9N2 viruses

Sample	Source	Virus	HA			NA		PB2	M1		M2	NS1
			Q226L	G228S	Cleavage site	R294K	Stalk deletion (positions)	E627K	D701N	N30D	T215A	S31N	P42S
hd4	Chicken feces	A/environment/Hangzhou/34-1/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	E	D	D	A	N	S
		A/environment/Hangzhou/34-2/2013(H9N2)	L	G	PSRSSR*GL	R	63–65						
hd6	Chicken pharyngeal swab	A/chicken/Hangzhou/48-1/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	E	D	D	A	N	S
		A/chicken/Hangzhou/48-2/2013(H9N2)	L	G	PSRSSR*GL	R	63–65						
hd7	Chicken pharyngeal swab	A/chicken/Hangzhou/50-1/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	E	D	D	A	N	S
		A/chicken/Hangzhou/50-2/2013(H9N2)	L	G	PSRSSR*GL	R	63–65						
hd11	Quail pharyngeal swab	A/quail/Hangzhou/35/2013(H9N2)	L	G	PSRSSR*GL	R	63–65	E	D	D	A	N	S
	Human	A/Hangzhou/1/2013(H7N9)	I	G	PEIPKGR*GL	R	69–73	K	D	D	A	N	S
	Human	A/Hangzhou/2/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	K	D	D	A	N	S
	Human	A/Zhejiang/DTID-ZJU01/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	E	N	D	A	N	S
	Chicken	A/chicken/Zhejiang/DTID-ZJU01/2013(H7N9)	Q	G	PEIPKGR*GL	R	69–73	E	D	D	A	N	S
	Human	A/Shanghai/1/2013(H7N9)	Q	G	PEIPKGR*GL	K	69–73	K	D	D	A	N	S
	Human	A/Shanghai/2/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	K	D	D	A	N	S
	Human	A/Anhui/1/2013(H7N9)	L	G	PEIPKGR*GL	R	69–73	K	D	D	A	N	S

sequences of the H9N2 viruses (A/chicken/Hangzhou/48–2/2013, A/chicken/Hanghzou/50–2/2013, and A/quail/Hangzhou/35/2013) from live poultry markets were clustered into relatively independent clades in their respective phylogenetic trees (Fig. 3). These H9N2 viruses belonged to a lineage that descended from the Ck/Bei-like or Y280/G9-like viruses (also designated lineage h9.4.2 in a new nomenclature) such as A/chicken/Hong Kong/G9/1997, A/chicken/Beijing/1/94, and A/duck/Hong Kong/Y280/1997. The closest hits for the H9 and N2 sequences according to the Basic Local Alignment Search Tool (BLAST) were H9 of A/chicken/Henan/HF/2012(H9N2) with similarities of between 98.4% and 98.7% and N2 of A/chicken/Guangdong/YBS10/2011(H9N2) with similarities of between 98.1% and 98.4%. A/chicken/Henan/HF/2012(H9N2) belongs to a sublineage of h9.4.2 viruses, h9.4.2.5, which has been found to be widely distributed in chickens in China in recent years (22).

In general, the six internal genes of the H9N2 viruses from live poultry markets were very close to the genes of the human H7N9 viruses [A/Hangzhou/1/2013(H7N9) and A/Hanghzou/2/2013(H7N9)] and the H9N2 viruses circulating in chickens in China recently (Fig. 4). However, the closest internal genes of the H7N9 viruses from live poultry markets came from different isolates of H7N9 viruses (Fig. 4), suggesting that the internal segments had multiple sources which might result from frequent reassortment among various genotypes of H9N2 virus or with other subtypes.

DISCUSSION

In this study, we comprehensively surveyed epidemiologically linked live poultry markets for 10 human cases in Hangzhou, China, and identified H7N9-positive specimens in all markets. Moreover, we found that the environments of these live poultry markets were heavily contaminated by both avian influenza virus subtypes H7N9 and H9N2, with or without H5N1. Furthermore, the coexistence of H7N9 and H9N2 virus was identified in chickens and ducks in one local market, indicating that the H7N9 virus not only probably originated from the segment reassorting with H9N2 virus but also was prevailing with H9N2 virus among the chickens and ducks in live poultry markets in Hangzhou at least during the period of emergence of the virus.

H9N2 viruses have prevailed in chickens in China in recent years and have constantly undergone reassortment, and novel genotypes have continued to emerge (21, 22, 23, 24). The H9N2 virus from live poultry markets in Hangzhou seemed to evolve from the H9N2 virus circulating in chickens in China recently on the basis of the high sequence similarities of all eight segments. The phylogenetic analysis revealed that the internal genes of H7N9 and H9N2 viruses from poultry in Hangzhou were of multiple origins, suggesting the prevalence of viruses with various genotypes of internal genes with ongoing reassortment in poultry.

However, it seems impossible that these 12 live poultry markets, most of them scattered throughout the urban area of Hangzhou, were the original places where H7N9 virus and H9N2 virus started to reassort, because there was little exchange of poultry among these markets. According to the information from the local agriculture department of the Hangzhou government, approximate 70% of poultry in Hangzhou markets came from two live poultry wholesale markets located in the suburbs, where poultry were imported from the Jiangshu province and Anhui province in which the early cases of human H7N9 infections were reported

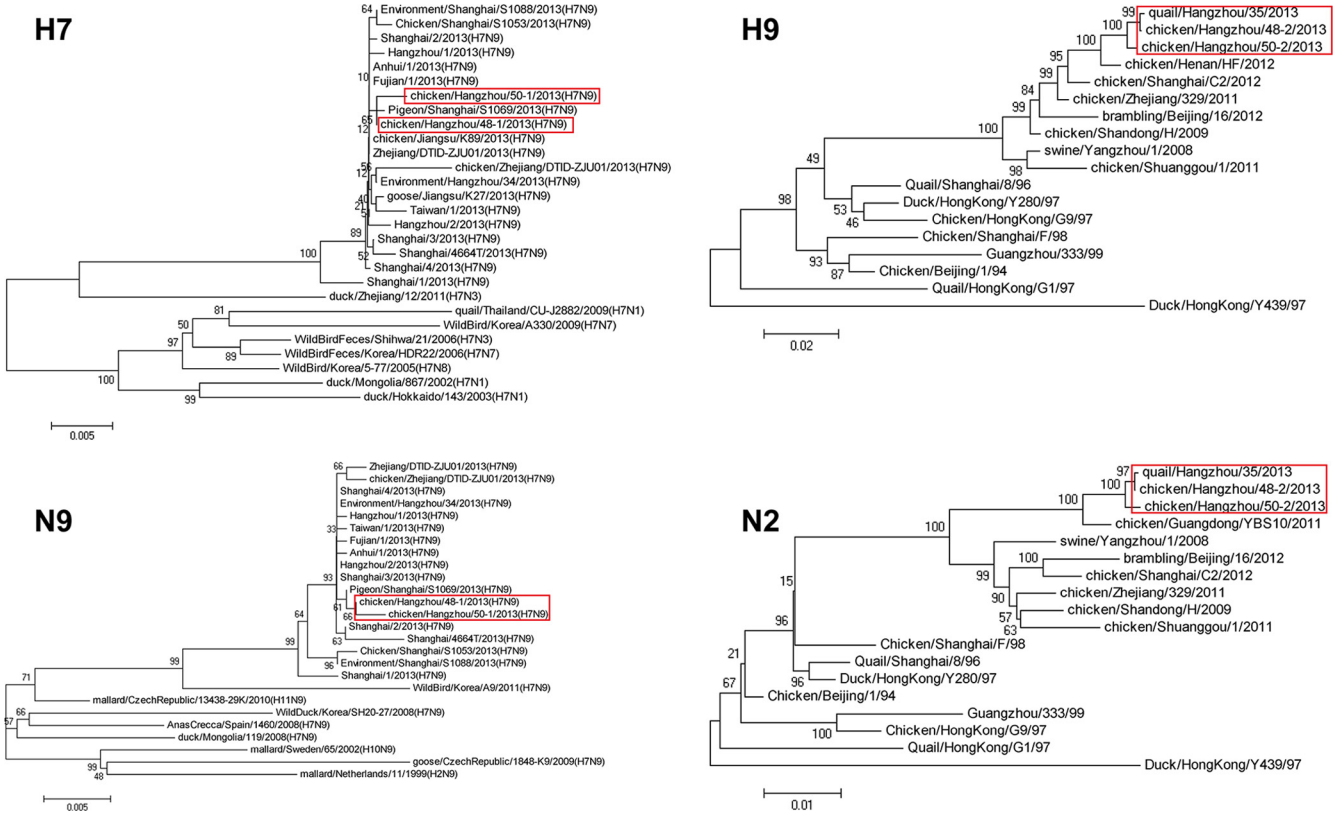

FIG 3 Phylogenetic analyses of hemagglutinin genes and neuramidinase genes of H7N9 viruses and H9N2 viruses. The viruses reported in this paper were highlighted using red frames.

(1). Thus, we propose a possible scenario of H7N9 emerging and causing human infection: (i) the H9N2 virus prevailing among chickens or ducks in China got H7 and N9 from other sources, probably in poultry farms or live poultry wholesale markets, and two subtypes started to coprevail; (ii) due to the fact that the H7N9 and the H9N2 viruses did not cause visible abnormal signs in poultry, two viruses were distributed with chickens or ducks to live poultry markets for retail, such as these 12 markets of Hangzhou, by live poultry trade systems; (iii) the environments of markets were heavily contaminated by H7N9 and H9N2 viruses; and (iv) a lot of people visited these markets every day, and then a few of them got infections.

With closure of the live poultry markets or warmer weather, human infections with avian influenza virus H7N9 came to an apparent halt in May 2013 in eastern China (from the weekly reports of H7N9 virus infection at the website of the National Health and Family Planning Commission of China). Recently, the live poultry trade has been restarted in Hangzhou, because no new human H7N9 infection had been reported for more than 2 months. Worries about resurgence of the H7N9 infections with the change of season remain. In this study, the live poultry markets in Hangzhou were highly contaminated with H7N9 virus during an outbreak of human H7N9 infections. Compared to the hundreds and thousands of people visiting these markets every day, the low number of human H7N9 infection cases suggests that the virus's ability of transmission from poultry to humans is still limited. So enhanced surveillance of the H7N9 virus or other subtypes in poultry would be helpful to provide an early warning of the H7N9 virus emerging. Moreover, it would minimize economic loss in agriculture to take control measures immediately after the H7N9 virus emerged in poultry rather than when human H7N9 infection was identified. However, the influence virus surveillance of poultry still remains poor in China. Closing live poultry markets in urban areas might be a good choice to reduce the risk of infection with the H7N9 viruses or the next novel subtype in the future. At that time, live poultry could be processed and frozen in factories, where virologic examination and biosecurity practices are easier to perform, and then could reach consumers safely.

The poultry markets were heavily contaminated by both H7N9 virus and H9N2 virus, suggesting that people might have the same chance to contact either of the viruses; however, only H7N9 viruses were detected in patients. This indicated that H7 and N9 played a key role in causing infections in human. The substitution of Q226L in H7 and the deletion of positions 69 to 73 in the N9 stalk were identified in these environmental H7N9 viruses, and both changes were associated with the viral adaption to mammals. Interestingly, the Q226L substitution and a similar NA stalk deletion were also found in these H9N2 viruses. For the H9N2 virus, it has been demonstrated that the NA stalk deletion with the combination of an HA cleavage site of PSRSSR*GL increased the virulence of virus in chicken and mice (21). The molecular basis of the H7N9 virus causing human infection needs to be explored further, and the trend indicating that the H9N2 virus is becoming more likely to infect humans directly should be closely monitored.

In summary, we provide direct evidence that H9N2 strains coexisted with the novel human-pathogenic H7N9 influenza virus

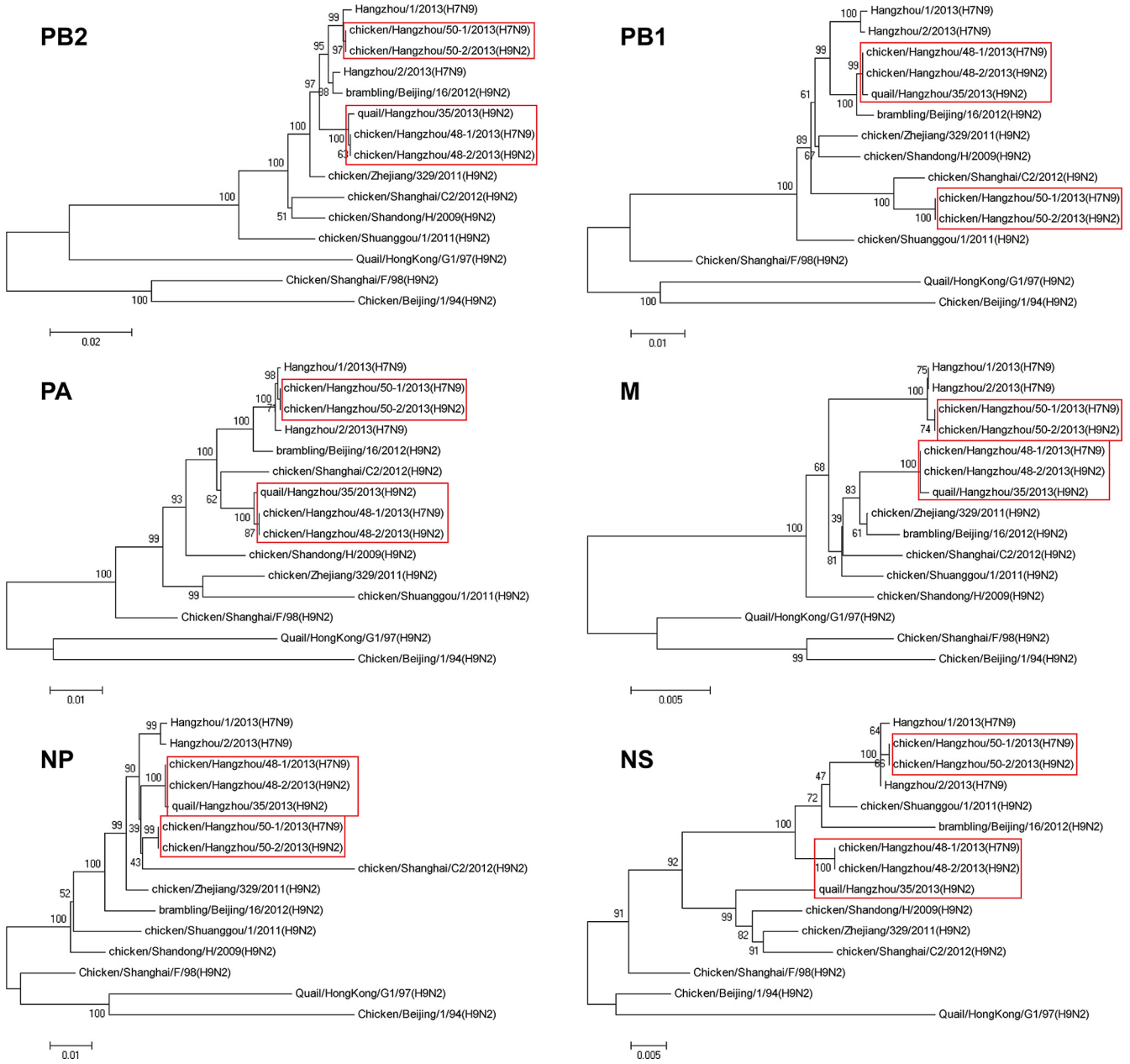

FIG 4 Phylogenetic analyses of six internal genes of H7N9 viruses and H9N2 viruses. The viruses reported in this paper were highlighted using red frames.

in epidemiologically linked live poultry markets, suggesting that avian influenza A virus of the H9N2 subtype likely made a recent contribution to the evolution of the H7N9 virus and that the contribution is ongoing.

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J. Pan, Jun Wang, R. Yang, and G. F. Gao designed and supervised the study as coprincipal investigators. X. Yu, X. Pu, J. Li, Y. Yu, Y. Kou, Y. Zhou, X. Wu, H. Wang, and J. Zhou did viral nucleic acid testing and analysis. D. Liu, S. Song, L. Xie, R. Huang, H. Ding, and G. Zhao performed collection and analysis of epidemiological data. T. Jin, J. Xu, G. Liu, H. Jia, Y. Wang, X. Xu, Y. Yin, and J. Wang did high-throughput next-generation sequencing and analysis. Y. Cui, C. Guo, X. Yang, and L. Hu did data analysis and prepared the figures. J. Pan, R. Yang, X. Yu, T. Jin, and H. Jia did data interpretation and wrote the manuscript.

We declare that we have no financial or commercial conflicts of interest.

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